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# Separation and Identification of Mangrove Condensed Tannin

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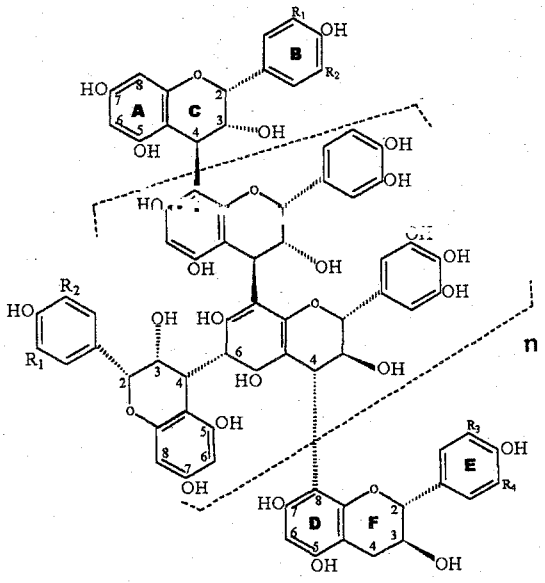
**Abstract** Finely ground mangrove barks (*Rhizophora apiculata*) was submitted to successive solid-liquid extractions to produce condensed tannin of high purity. Depolymerization of condensed tannin in the presence of phloroglucinol nucleophiles in acidic dioxane and acidic ethanol were carried out. The separation of flavan-3-ols and their phloroglucinol adducts using reversed phase high performance liquid chromatography is reported. The results showed that the condensed tannin is constituted of predominantly an extender unit and three terminal units, two of which have been identified as (+)-catechin and (-)-epicatechin.

## Introduction

Proanthocyanidins (condensed tannins) are a group of phenolic polymers which are widely distributed in the plant kingdom particularly in plants with woody growth habit. These compounds consist of flavan-3-ol units linked together through C4-C6 or C4-C8 bonds (fig. 1). Structural variation of proanthocyanidins ranges from dimers and trimers to more complex oligomers and polymers depending on the nature of the interflavanoid lingkage, hydroxylation pattern and stereochemistry at the three chiral centres (carbon 2,3 and 4) of the C-ring. They are the most widespread polyphenols in plants after lignins and can be found in leaves, fruits, woods, barks or roots and are known to play various nutritional and ecological roles.



Extender Unit "ACB" :
Epicatechin : $R_1$=OH, $R_2$=H
Epigallocatechin : $R_1$=$R_2$=OH
Epiafzelechin : $R_1$-$R_2$-H

Terminal Unit "DFE" :
Catechin : $R_3$=OH, $R_4$=H
Gallocatechin : $R_3$=$R_4$=OH
Afzelechin : $R_3$=$R_4$=H

Fig. 1 : Structure of a condensed tannin

Several methods have been proposed for their estimation [1]. The most largely used are colorimetric assays. Other depolymerization reactions in the presence of nucleophiles are frequently employed for the structural analysis of proanthocyandins. The nucleophile forms adducts with the extender units in the polymer, which are purified or analyzed by chromatography. Their structure, once established, can be used to determine the nature of the monomer units within the polymer. Numerous nucleophiles have been used including benzylthiol[2], benzyl mercaptan [3,4] or phrologlucinol[5-9]. Depolymerization in the

presence of a nucleophile offers several advantages. Associated with the quantitative analysis of the products by chromatography, it may simultaneously offer information on the nature of the proanthocyndins. In contrast to colorimetric methods, interferences with other plant constituents are avoided due to the nonambiguous identification of the proanthocyanidin-derived products. In comparison to the butanol/HCL depolymerization, the reaction preserves the stereochemistry at the C2-C3 positions of the polymer units. The use of a nucleophile limits the occurrence of side reactions that could affect recovery yields of the products.

The aim of our work is to separate and characterize for the first time, the mangrove (*Rhizophora appiculata*) condensed tannin by a depolymerization reaction in the presence of a phloroglucinol nucleophile and its products analysed by reversed phase HPLC, leading to a quantitative determination of condensed tannin content in future.

**Experimental**

*Tannin isolation*

The extraction of tannin from mangrove barks (*Rhizophora apiculata*) was carried out by total immersion of finely ground barks in 70% aqueous acetone for 72 hours at room temperature. The acetone was removed under pressure and the resulting aqueous fraction was freeze-dried, yielding 25-27% weight of the dry barks. The residual aqueous phase (1.5 g) was defatted with hexane, followed by the extraction with ethyl acetate and the aqueous phase was freeze-dried. A fraction of the aqueous phase (1.0 g) was dissolved in methanol/water 1:1 and loaded onto a Sephadex LH20 column. The column was washed with methanol/water 1:1 to eliminate sugars and sugar-containing proanthocyanidins and was eluted with acetone/water 1:1 to produce condensed tannin. The methanol/water and acetone/water fractions were reloaded onto the Sephadex LH20 column and the elution procedure repeated to determine the purity of the extractions. A Perkin Elmer System 2000 was used to obtain the FTIR spectrums.

*Phloroglucinol Degradation*

Method 1. Condensed tannin (25 mg), phloroglucinol (8 mg) and dioxane/aqueous HCL 0.2 M, 1:1 (0.5 mL) were added in a tube which was sealed and heated at 80°C for 20 minutes. An aliquot was then diluted exactly with methanol/water 1:1.

Method 2. Condensed tannin (0.01 g) was dissolved in 1.5 mL of the phloroglucinol solution (5 gm/mL phloroglucinol in acidic ethanol) and allowed to react at room temperature overnight. The solvent was then evaporated under nitrogen, and the residue dissolved in 0.5 mL distilled water. This solution was extracted three times with ethyl acetate (1.5 mL per extraction). The three ethyl acetate fractions were combined and evaporated under nitrogen. The residue was dissolved in 1.0 mL of 70% aqueous methanol. The procedure was repeated using (+)-catechin, (-)-epicatechin and methanol/water fraction samples.

*HPLC analysis*

Degradation products were analysed on a Varian Prostar , utilizing a Crestpak C18S column, at a flow-rate of 1 mL/min and detected at 280 nm using a UV detecter. The elution conditions for method 1 were as follows: Solvent A, water/phosphoric acid 999 : 1; solvent B, methanol; linear gradient 0-90% B in 30 minutes. Two elution conditions were used for method 2 : (i) Solvent A, 1% aqueous acetic acid; solvent B, methanol; 0-30 minutes, 0-15% B in A (linear gradient); 30-45 minutes 15-60% B in A (linear gradient); 45-50 minutes, 60% B in A (isocratic). (ii) Solvent A, 1% aqueous acetic acid; solvent B Methanol : Solvent A, 60 : 40 (v/v); t=0, 100% solvent A; t=60 minutes, 40% solvent A, 60% solvent B; t= 65 min, 100% solvent B. The phloroglucinol, (+)-catechin and (-)-epicatechin standards prepared in 70% aqueous methanol were used to identify the peaks.

*Determination of condensed tannin*

The determination of the condensed tannin based on catechin equivalent for the acetone/water and methanol/water extracts were carried out using the vanillin assay as described by Price et. al [10]

**Results and Discussion**

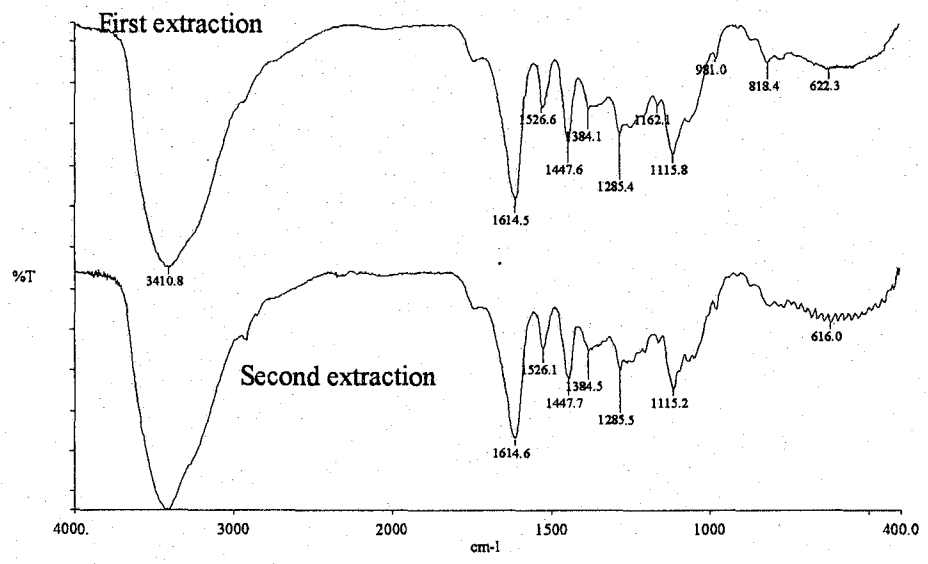



Fig. 2 : FTIR spectrum of condensed tannins

The FTIR spectrum of a condensed tannin is shown in fig. 2. A very broad absorption band between 3,700 and 2,700 is due to the presence of hydroxyl groups. Three peaks occurring at 1,614, 1,521 and 1,447 are characteristic of aromatic compounds. Various peaks in the 600 to 900 cm-1 and smaller peaks between 1,000 and 1,200 cm-1 are characteristic of substituted benzene rings. All the major peaks shown are typical peaks of a condensed tannin as observed by Nasrazadini[11]. The spectrum corresponding to the second extraction sample gave rise to similar major peaks.

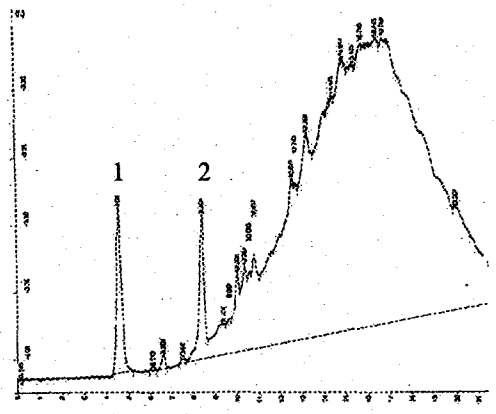



Fig. 3 : HPLC chromatogram of the condensed tannin degraded in the presence of phloroglucinol and acidic dioxane.

In the acid depolymerization in the presence of phloroglucinol, the interflavan bonds are protonated and broken, leaving the terminal unit intact and the extender unit as a carbocation. The carbocation is then captured by the phloroglucinol either alpha or beta to the C-ring (fig. 1) at C-4, producing a monomer-phloroglucinol adduct. When the condensed tannin was submitted to acid degradation in the presence of phloroglucinol and dioxane, two main narrow peaks and an intense broad peak were observed (fig. 3). The two narrow peaks were identified as being phloroglucinol and phloroglucinol adduct. The broad peak could be attributed to the fraction resistant to the depolymerization [4].

When the condensed tannin was submitted to acid degradation in the presence of phloroglucinol and ethanol, five narrow peaks followed by a hump were observed (fig. 4a). However when another elution condition was adapted as described in method (ii) of the HPLC analysis, the HPLC chromatogram as shown in fig. 4(b) was obtained, showing well resolved peaks with no hump. Comparison of the retention times and spiking the samples with the (+)-catechin and (-)-epicatechin standards confirmed the presence of these monomers as terminal units in the mangrove condensed tannin. The retention times for the various acid depolymerizations and different elution conditions used are as given in table 1.

Table 1 : Retention times of some flavan-3-ol and phloroglucinol adduct of the different phloroglucinol degradation reactions and different elution conditions.

| | Method 1 | Method 2 Elution condition (i) | Method 2 Elution condition (ii) |
|---|---|---|---|
| | $t_R$ (min) | $t_R$ (min) | $t_R$ (min) |
| 1. Phloroglucinol | 4.50 | 3.53 | 3.48 |
| 2. Phloroglucinol adduct (unknown) | 8.41 | 12.28 | 11.48 |
| 3. (+)-catechin | - | 22.01 | 18.87 |
| 4. (-)-epicatechin | - | 34.62 | 29.20 |
| 5. Unknown terminal unit | - | 37.24 | 36.10 |

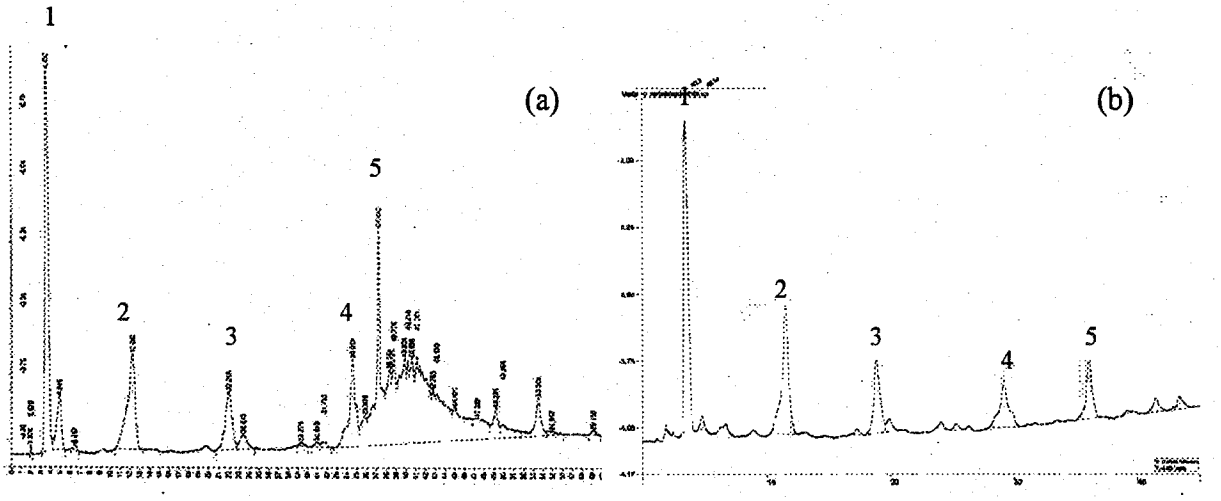



Fig. 4 : HPLC chromatogram of the condensed tannin degraded in the presence of phloroglucinol and acidic ethanol using the (a)elution condition (i) and (b) elution condition (ii)

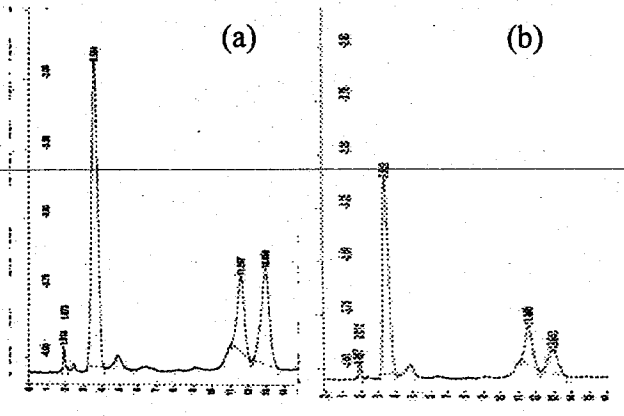



Fig. 6 :HPLC chromatogram when spiked with the adducts of (a)(+)-catechin and (b) (-)-epicatechin

Due to the unavailability of phloroglucinol adducts commercially, the identity of the adduct could not be ascertained. However when the sample was spiked with catechin and epicatechin adducts synthesized in the lab, the HPLC chromatograms (fig. 5a & 5b) showed a pair of phrologlucinol adducts indicating the absence of catechin and epicatechin extender units in the mangrove condensed tannin. Thus, three terminal units and one extender unit were separated in the acid depolymerization mixture from the mangrove bark samples. We are in the process of identifying the phloroglucinol adduct and the third terminal unit. The well resolved peaks of the HPLC chromatogram

(fig. 4b) will facilitate the preparative HPLC analysis in identifying these fractions.

A quantitative measurement of the peak areas showed that the ratio of catechin:epicatechin:unknown terminal unit is 0.38:0.34:0.28. The highest concentration of catechin in the mangrove condensed tannin further affirms our choice of standard used for the determination of condensed tannin as catechin equivalent using the vanillin assay. The spectrometric quantifications yielded a total content of 34-38 % (in (+)-catechin equivalent) in the mangrove bark. Among the weaknesses associated with the vanillin assay is the lack of specificity for condensed tannin. Any appropriately substituted monomeric flavanol reacts in the assay. The major problem with the vanillin assay seems to derive from the variable reactivity of the subunits of the tannin polymer. The structural variations in proanthocyanidins also affects the colour yield with vanillin[1]. In view of these weaknesses, the HPLC analysis following depolymerization in phloroglucinol could therefore provide an alternative method to quantitatively measure the condensed tannin content.

Based on the vanillin assay conducted, no difference was noted in the catechin content between the first and second extracted samples of the condensed tannin. On the other hand, the HPLC analysis, proving to be a more sensitive tool, showed a small increase of 8-10 % in the catechin peak area when the condensed tannin was extracted for the second time. It was also discovered that a small amount of condensed tannin were present in the methanol/water extract as shown from the vanillin assay and HPLC chromatogram. The HPLC chromatogram showed peaks similar to that of condensed tannin. According to the vanillin assay, the catechin equivalent in the methanol/water extract was found to be 4.8-6.1%. Any attempt to further separate the condensed tannin from the methanol/water extract would prove to be a futile effort since the second methanol/water fraction again showed the presence of condensed tannin when subjected to vanillin assay and HPLC analysis. This is in agreement with the work reported by Ahmadi et. al.[8].

**Conclusion**

1. The method of extraction used produced condensed tannin of high purity without further purification.
2. Depolymerization of the condensed tannin in the presence of phloroglucinol in acidic ethanol, followed by HPLC analysis gave rise to well resolved peaks corresponding to one extender unit and three terminal units.
3. Two of the terminal units of the mangrove condensed tannin were identified as (+)-catechin and (-)-epicatechin.
4. The HPLC analysis following depolymerization in phologlucinol could provide an alternative procedure for the quantitative measurement of condensed tannin.

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**References**

1. Schofield,P. Mbugua, D.M. and Pell, A.N. *Animal Feed Science and Technology*, **2001**, 91, 21-40.
2. Foo,L.Y. and Karchesy,J.J. *Phytochemistry*. **1991**, 30(2), 667-670.
3. Guyot, S.; Doco, T.; Souquet, J.M.; Moutounet,M. and Drilleau, J.F. *Phytochemistry*, **1997**, 44(2), 351-357.
4. Matthews,S.; Mila, Scalbert, A.; Brigitte, P.; Lapierre, C.; Herve du Penhoat, C.L.M.; Rolando, C. and Donnelly, D.M.X. *J. Agric. Food Chem.*, **1997**, 45, 1195-1201.
5. Prieur, C.; Rigaud, J.; Cheynier, V. and Moutounet, M. *Phytochemistry*, **1994**, 36(3), 781-784.
6. Brandon, M.J.; Foo, L.Y.; Porter, L.J. and Meredith, P. *Phytochemistry*, **1982**, 21(12), 2953-2957.
7. Koupai-Abyazani, M.R.; McCallum and Bohm, B.A. *J. Chromatography*, **1992**, 594, 117-123.
8. Ahmadi, S.; Shahbirin, G.; Choong, E.T. and Hemingway, R.W. *Phytochemistry*, **1994**, 36(1), 217-219.
9. Hernes, P.J.; Benner, R.; Cowie,G.L.; Goni, M.A.; Bergamashi, B.A. and John I.H. *Geochimica et Cosmochimica Acta*, **2001**, 65(18), 3109-3122.
10. Price,M.L.; Scoyoc, A.V. and Butler, L.G. *J.Agric. Food Chem.*, **1978**, 26, 1214-1218.
11. Nasrazadini, S. *Corros.Sci.*, **1997**, 39(10-11), 1845-1859.